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Review

Structural Insights into the Mechanisms of Antibody-Mediated Neutralization of Flavivirus Infection: Implications for Vaccine Development

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Flaviviruses are a group of small RNA viruses that cause severe disease in humans worldwide and are the target of several vaccine development programs. A primary goal of these efforts is to elicit a protective humoral response directed against the envelope proteins arrayed on the surface of the flavivirus virion. Advances in the structural biology of these viruses has catalyzed rapid progress toward understanding the complexity of the flavivirus immunogen and the molecular basis of antibody-mediated neutralization. These insights have identified factors that govern the potency of neutralizing antibodies and will inform the design and evaluation of novel vaccines.

Flaviviruses are a group of ~70 positive strand RNA viruses, many of which are transmitted through the bite of an infected mosquito or tick and cause a variety of severe diseases in humans (Lindenbach et al., 2007). Dengue virus (DENV) is a mosquito-borne flavivirus endemic to tropical and subtropical regions of the world, with ~30% of the human population at risk of infection. Four antigenically related serotypes of DENV circulate in nature and are responsible for more than 50 million human infections each year (Kyle and Harris, 2008). While the majority of these infections are inapparent, clinical manifestations range from a self-limited febrile illness to a potentially fatal disease characterized by hemorrhage (dengue hemorrhagic fever; DHF) and/or shock (dengue shock syndrome; DSS) (Gubler, 1998). The incidence of DHF/DSS has increased significantly during the past 50 years and is due, in part, to the global spread of multiple DENV serotypes (Kyle and Harris, 2008). Other members of this genus with a major impact on public health include yellow fever virus (YFV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), and West Nile encephalitis virus (WNV).

In light of the global clinical and economic burden of dengue infection, the development of a vaccine is being actively pursued by both the private and public sector (reviewed by Whitehead et al., 2007). Based on past achievements with other flaviviruses and recent scientific advances in understanding dengue biology, there is cause for optimism that these efforts will yield a vaccine capable of protecting against DENV infection. Safe and effective vaccines that prevent infection by other flaviviruses have been developed including the live-attenuated 17D vaccine for YFV (Monath, 2005), an inactivated TBEV vaccine (Heinz and Kunz, 2004), and both live-attenuated and inactivated JEV vaccines (Hennessy et al., 1996; Kurane and Takasaki, 2000; Xin et al., 1988). Altogether, these have been administered to more than 400 million individuals, with relatively few (albeit in some cases serious) adverse events (Monath, 2007). These successful vaccine efforts have established the immunogenicity of flaviviruses in humans, facilitated an understanding of surrogate markers of protection, and identified strategies and vectors capable of eliciting protective responses. Finally, as the immune response elicited by natural DENV infection confers life-long protection against reinfection by viruses of the same serotype, vaccination and immunologic protection against DENV should be feasible (Whitehead et al., 2007).

The development of a DENV vaccine, however, is complicated by a requirement to protect simultaneously against the four serotypes of DENV and the potential for a suboptimal vaccine-induced immune response to exacerbate disease. Prospective clinical studies suggest that the risk of severe disease is significantly greater for individuals experiencing DENV infection for the second time with a heterologous DENV serotype (Vaughn et al., 2000). The viral and host factors that contribute to the development of severe DENV disease following secondary infection remain controversial and are an area of intensive study (Green and Rothman, 2006; Halstead, 2003). A central role for DENV-reactive antibody in initiating the pathogenesis of severe disease is strongly suggested by the finding that infants of DENV-immune mothers are at increased risk for DHF/DSS following primary infection during their first year of life (Chau et al., 2008; Kliks et al., 1988). In this context, passively transferred maternal antibody increases the severity of disease, presumably by promoting more efficient infection of Fc-γ-receptor-expressing myeloid cells in vivo: this phenomenon is called antibody-dependent enhancement of infection; ADE) (Halstead and O'Rourke, 1977).

The potential for vaccine-induced antibody responses to protect against infection or exacerbate disease highlights the need to understand, in structural and biochemical detail, the complexity of the humoral immune response against flaviviruses, including DENV. Over the past few years, rapid progress has

been made in elucidating the factors that govern the potency of anti-flavivirus antibodies. These efforts have been catalyzed in part by support from the Research and Development Network of the Pediatric Dengue Vaccine Initiative (PDVI; http://www.pdvi.org), a Bill and Melinda Gates Foundation-sponsored global network of basic science and clinical investigators. This Review will discuss the molecular basis of antibody-mediated neutralization and enhancement of flavivirus infection and how these concepts inform the rational design and evaluation of vaccines, therapeutics, and diagnostics.

The Critical Role of Antibodies in Protection against Flavivirus Infection

Humoral immunity is a component of the host response to flaviviruses that is essential to control dissemination of infection, and for encephalitic flaviviruses, to arrest viral replication in the central nervous system. Passive transfer of polyclonal or monoclonal antibodies (mAbs) against flavivirus proteins protects mice against lethal challenge in several different flavivirus systems (reviewed in Pierson and Diamond, 2008 and Roehrig et al., 2001). Furthermore, mice that lack B cells are more vulnerable to flavivirus infection (Chambers et al., 2008; Diamond et al., 2003; Halevy et al., 1994). Antibodies are believed to protect against flavivirus infection through multiple mechanisms, including (1) direct neutralization of receptor binding, (2) inhibition of viral fusion, (3) Fc-γ-receptor-dependent viral clearance, (4) complement-mediated lysis of virus or infected cells, and (5) antibody-dependent cytotoxicity of infected cells.

Flavivirus Structure

Flaviviruses are relatively small (~50 nm diameter) spherical virions that encapsidate a nonsegmented RNA genome of positive-sense polarity (Lindenbach et al., 2007). This ~11 kb viral RNA encodes a single polyprotein, which is cleaved by viral and cellular proteases into ten functionally distinct proteins (three structural and seven nonstructural proteins). Virions are composed of the capsid (C), premembrane/membrane (prM/M) and envelope structural proteins (E), the viral RNA, and a lipid envelope derived from the endoplasmic reticulum (ER).

The structure of the ectodomain of the E protein of several flaviviruses has been resolved by X-ray crystallography and is composed of three distinct domains connected to the viral membrane by a helical stem anchor and two antiparallel transmembrane domains (reviewed in Mukhopadhyay et al., 2005). Domain III (E-DIII) adopts an immunoglobulin-like fold and has been suggested to play a critical role in receptor interactions. Domain II (E-DII) is composed of two long finger-like structures and contains a highly conserved 13 amino acid fusion loop (E-DII-FL). The central domain of E (domain I; E-DI) is a nine-stranded β-barrel that is connected to E-DIII and E-DII by one and four flexible linkers, respectively (Figure 1A). E proteins are important for viral assembly, receptor attachment, entry, viral fusion, and possibly immune evasion during the flavivirus life cycle and, thus, are dynamic proteins required to adopt several distinct conformations and arrangements on the virus particle (Arjona et al., 2007; Mukhopadhyay et al., 2005). Moreover, the primary target for neutralizing anti-flavivirus antibodies is the E protein (Roehrig, 2003).

Flaviviruses assemble at the endoplasmic reticulum (ER) and bud into the lumen as immature virus particles in which three

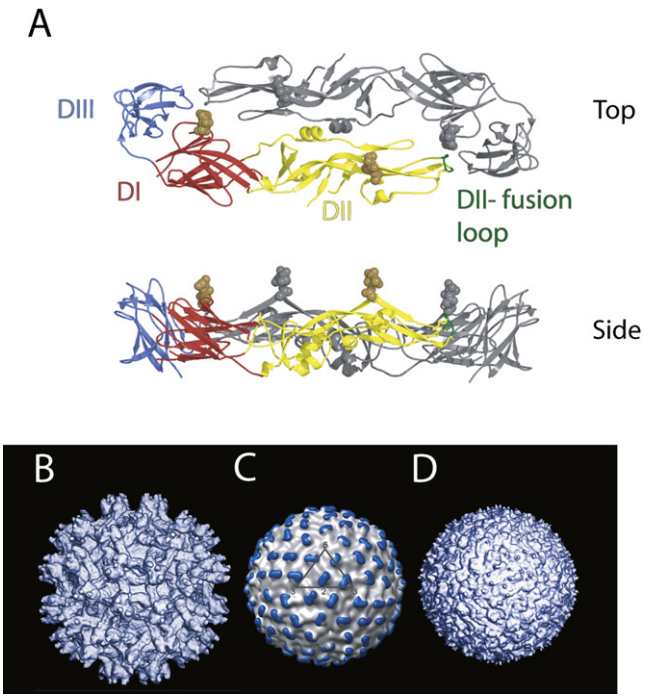

Figure 1. Structure of the DENV E Protein and Its Arrangement on the Virion

(A) Ribbon diagram of the antiparallel DENV E protein dimer as seen from the top and side. Individual domains of the E protein are indicated, as is the fusion loop at the distal end of E-DII. The N-linked carbohydrate modifications on E are shown as brown spheres

(B–D) Surface shaded representation of the DENV virion at varying stages of maturation as determined by cryo-EM reconstruction. The significant conformational differences in the organization of E proteins of the virion during maturation are evident by comparing structure of spiky immature virions at (B) neutral and (C) acidic pH. In the latter case, prM is represented in cyan and the icosahedral asymmetry unit is indicated as a gray triangle. In (D), the structure of the mature DENV virion is shown. The images presented in (B)–(D) were created using previously described structures of DENV (Kuhn et al., 2002; Yu et al., 2008; Zhang et al., 2003).

pairs of E and prM interact as trimers arranged as sixty spiked projections with icosahedral symmetry (Figure 1B) (Zhang et al., 2003, 2007). The E proteins of each trimer project away from the surface of the virion and interact with prM via the distal end of E-DII including the fusion loop (Li et al., 2008). PrM on immature virions restricts the ability of E proteins to undergo oligomeric rearrangement in the low pH Golgi-derived secretory compartments during viral egress, thus preventing premature and adventitious fusion (Guirakhoo et al., 1991; Heinz et al., 1994). As immature virions traffic through the acidic compartments of the trans-Golgi network (TGN), changes in the orientation of prM and E proteins unmask a site for the cellular serine protease furin (Stadler et al., 1997; Yu et al., 2008). In this low pH environment, the E proteins of immature virions form antiparallel dimers that lie flat against the surface of the virion and are arranged with T = 3 quasi-icosahedral symmetry (Figure 1C) (Yu et al., 2008). The prM protein continues to mask the fusion loop of E-DII until it is released after furin cleavage and a transition to neutral pH occurs in the extracellular space. The resulting mature and infectious viruses are relatively smooth particles composed of 90 E protein dimers and 180 copies of the ~70 amino acid M protein.

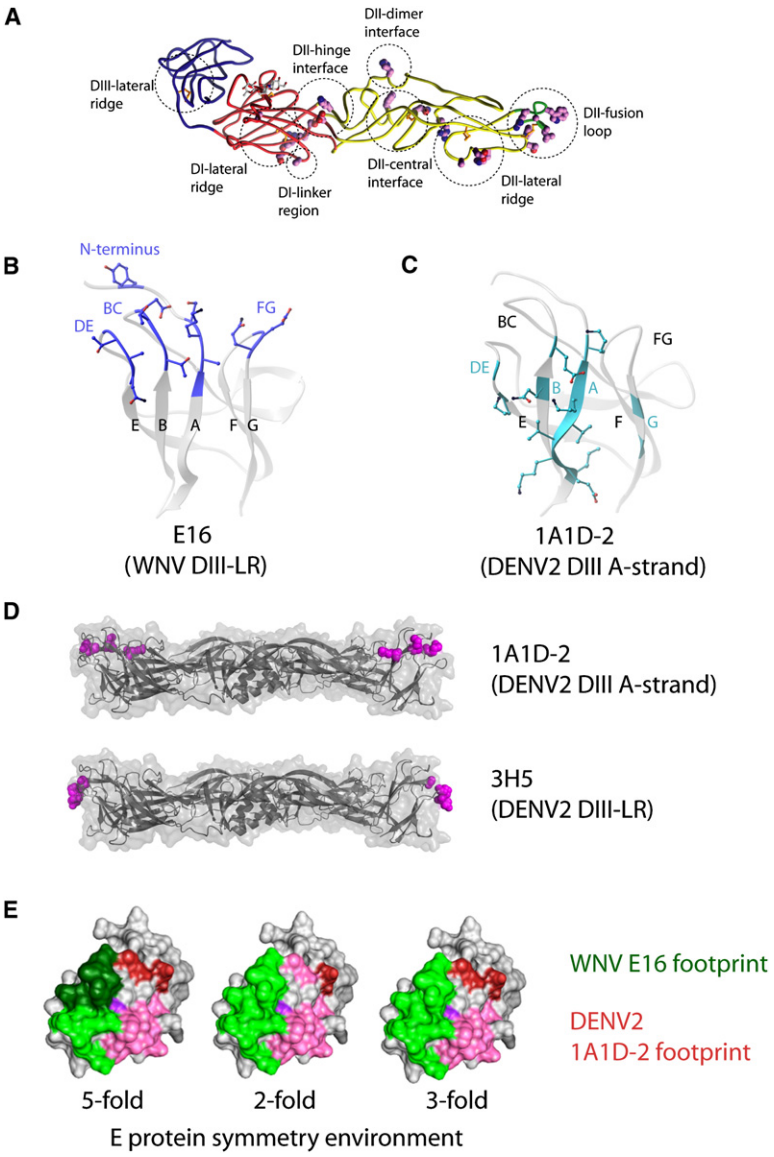

Figure 2. Antigenic Complexity of the Flavivirus E Protein

(A) All three domains of the E protein contain epitopes recognized by neutralizing antibodies. Domains of the WNV E protein are labeled as in Figure 1. Residues that impact antibody binding (shown in magenta) were identified using a yeast-display mapping approach for a large panel of antibodies, identifying structurally distinct epitopes in E-DIII (below), E-DI, and E-DII. Epitopes are labeled as described previously (Oliphant et al., 2006).

(B) The structure of WNV DIII is shown displaying the epitope recognized by the type-specific mAb E16. Residues of E-DIII determined by X-ray crystallography to interact directly E16 are highlighted in blue.

(C) The structure of DENV E-DIII is shown displaying the epitope recognized by the sub-complex-specific mAb 1A1D-2 (highlighted in cyan). Note that the epitope for 1A1D-2 is centered on the A strand and does not make contact with the residues in the FG loop that play a role in type-specific reactivity.

(D) Comparison of the epitope recognized by 1A1D-2 and the DENV2 type-specific mAb 3H5.

(E) E proteins exist on the mature flavivirus associated with three distinct symmetry environments. Epitopes recognized by mAbs may not be equally accessible in all three of these environments. The WNV-specific mAb E16 binds an epitope (shown in green) that is accessible in only two of these environments. As shown in dark green, the E-DIII-LR epitope recognized by E16 is partially obscured on E proteins proximal to the five-fold axis of symmetry, which limits binding to 120 of 180 possible E proteins on the mature virus. The 1A1D-2 epitope (shown in pink) on E-DIII is partially blocked on E proteins of mature virus particles regardless of their position on the virions (shown in red).

In this configuration, E proteins on the mature flavivirus exist in three distinct environments defined by their proximity to the 2-, 3-, or 5-fold axis of symmetry (Kuhn et al., 2002; Mukhopadhyay et al., 2003) (Figure 1D).

The Antigenic Structure of Flaviviruses

Epitopes recognized by neutralizing antibodies have been identified in all three domains of the E protein; however, many of the most potent mAbs are virus-type specific and bind epitopes within E-DIII (Figure 2A) (reviewed in Gromowski and Barrett, 2007; Roehrig, 2003; Sukupolvi-Petty et al., 2007 and references within). One of the well-characterized neutralizing mAbs, the anti-WNV E16 antibody, binds four discontinuous loops on the lateral ridge of WNV E-DIII (E-DIII-LR). E16 blocks WNV infection at picomolar concentrations in vitro (Oliphant et al., 2005; Pierson et al., 2007) and can protect animals against lethal challenge even when administered with a single dose up to 6 days after infection (Morrey et al., 2007). Structural and biochemical studies identified four amino acids (306, 307, 330, and 332) on the amino-terminal and BC loops as the core residues of the interface of E-DIII and E16; these residues are not conserved among flaviviruses, which explains the type specificity of this mAb (Figure 2B) (Nybakken et al., 2005). Molecular docking and cryoelectron microscopy (cryo-EM) studies reveal that E16 cannot bind all 180 E proteins on the virion (Kaufmann et al., 2006). Epitopes on the tightly clustered E proteins along the five-fold axis of symmetry remain unoccupied due to steric conflicts. These studies indicate that, at maximum, 120 E-DIII-LR epitopes on the mature virion can simultaneously bind E16. Similarly, some strongly neutralizing type-specific mAbs that neutralize DENV serotype 2 (DENV2) (e.g., mAb 3H5) also map to the E-DIII-LR; however, the epitope recognized by these mAbs is located on both the A strand (amino acid 304) and the FG loop (residues 383 and 384) (Figure 2D) (Sukupolvi-Petty et al., 2007). Not all antibodies that bind E-DIII exhibit type-specific neutralizing activity. The subcomplex-specific mAb 1A1D-2 recognizes an epitope centered on the A strand of the lateral surface of E-DIII and can neutralize infection by DENV serotypes 1–3, but not DENV4 (Lok et al., 2008; Roehrig et al., 1998; Sukupolvi-Petty et al., 2007). The molecular basis for the specificity of this mAb has been investigated; only one of three residues at the center of the 1A1D-2 epitope is conserved among all four DENV serotypes (Figure 2C). A similar A-strand epitope is also recognized by the broadly neutralizing crossreactive DENV mAb 4E11 (Thullier et al., 2001). Finally, E-DIII epitopes recognized by WNV- and DENV-specific mAbs with modest neutralizing

activity have been characterized. Typically, these map to residues that are less accessible on mature virions (Gromowski et al., 2008; Oliphant et al., 2005; Pierson et al., 2007; Roehrig et al., 1998; Sukupolvi-Petty et al., 2007). Altogether, structural and biochemical approaches identify E-DIII as a complex immunogen that elicits antibodies with varying specificity and functional potency.

Analysis of the B cell repertoire of flavivirus-immune humans suggest that many antibodies produced in vivo recognize the E-DII-FL (Throsby et al., 2006). Fusion loop-specific mAbs exhibit modest neutralization potency in vitro, protect animal models from WNV infection only at relatively high concentration, and are often crossreactive (Crill and Chang, 2004; Goncalvez et al., 2004; Nelson et al., 2008; Oliphant et al., 2006; Stiasny et al., 2006). The structural basis for the recognition of the E-DII-FL is not yet clear and may differ among mAbs. Structural models of the mature flavivirus virion do not provide a clear rationale to explain mAb recognition of intact virus particles because the fusion loop of each E protein is partially buried at the E-DI and E-DIII interface of the opposing E protein in the antiparallel dimer (Kuhn et al., 2002) (Figure 1A). Studies with TBEV demonstrate that treatment with mild nonionic detergent significantly increases E-DII-FL mAb binding to intact virions, suggesting accessibility of the E-DII-FL epitope is limited on mature viruses (Stiasny et al., 2006). However, the molecular details of mAb binding to this epitope may not be explained by static models of mature virion structure as described in detail below. Furthermore, the E-DII-FL epitope may be complex, composed of residues in other loops on the same E protein or residues on adjacent E proteins within the dimer (Crill and Chang, 2004).

A Moving Target …

Molecular docking of the crystallographic coordinates that define the E protein-antibody interaction onto a pseudoatomic model of the mature flavivirus has not always provided a clear structural basis for antibody recognition and neutralization. Indeed, some antibodies recognize "cryptic" determinants in the mature virion, such as the E-DII-FL, yet still exhibit some neutralizing activity; this suggests additional complexity not captured by static models of virion structure (Nelson et al., 2008; Stiasny et al., 2006). The cryo-EM approaches used to develop the existing models of virion structure identify the most abundant physical state of the virus particle and may fail to detect low abundance conformations that exist in a dynamic equilibrium. Recent cryo-EM experiments suggest that some antibodies may neutralize flaviviruses by recognizing different states of the E proteins on the virion. Molecular docking studies indicated that a significant portion of the 1A1D-2 Fab binding surface (~18%) is buried on E proteins present along all three symmetry axes of mature DENV virions (Figure 2E), consistent with poor binding of Fab molecules to the virion at 4°C (Lok et al., 2008). Incubation at physiologic temperatures resulted in Fab binding and significant changes in the arrangement of the E proteins on the surface of DENV; this allowed docking of 1A1D-2 Fab fragments to two of three E proteins in the asymmetric unit (Lok et al., 2008). The temperature dependence of binding suggests the 1A1D-2 mAb stabilized a state present in the ensemble of possible conformations of the virion at 37°C but not 4°C. How many different states exist in the ensemble of structures sampled by flavivirus virions, and the dynamics that regulate their relative abundance, is not presently known.

Engagement of antibodies that recognize low abundance, or transient, conformations of the E protein may alter the dynamics that govern the ensemble of conformations sampled by the virion. This in turn may modulate the efficiency of subsequent antibody-virion interactions. For 1A1D-2, the binding of Fab fragments to individual virions appeared to be an "all or none" phenomenon; that virions were either completely decorated by Fab or intact and unbound, suggested that the binding of a few Fab fragments might increase the accessibility of the epitope on surrounding E proteins in a cooperative fashion (Lok et al., 2008). In the context of a polyclonal response, changes in the accessibility of one epitope arising from the stabilization of E proteins in alternative conformations by antibodies specific for a distinct epitope adds to the complexity of understanding the humoral response in mechanistic and quantitative terms.

A Heterogeneous Target …

Cleavage of the prM protein by furin is a required step in the flavivirus life cycle (Mukhopadhyay et al., 2005). However, this maturation process can be inefficient, resulting in the release of virions that incorporate uncleaved prM protein yet remain infectious (Davis et al., 2006; Guirakhoo et al., 1992). Little is known about the extent of prM cleavage required for infectivity nor the arrangement of E protein on "partially mature" virions that incorporate prM protein. The release of heterogeneous populations of virions in vivo that differ with respect to the extent of prM cleavage may be an additional layer of complexity that shapes the repertoire of epitopes available for antibody binding and neutralization. Indeed, antibodies interact differently with the E protein trimers on immature particles compared to antiparallel dimers on fully mature virions (Guirakhoo et al., 1992; Heinz et al., 1994). As such, virion maturation impacts the sensitivity of flaviviruses to neutralization by some, but not all, antibodies (Nelson et al., 2008). Antibodies that recognize epitopes predicted to be poorly or partially exposed on mature virions were significantly less potent when assayed using a relatively homogeneous populations of mature virions, as compared to the heterogeneous population of virus particles typically released from cells. This reduced sensitivity of mature virions to neutralization may be explained by the inability of some classes of antibodies to engage the virion with a stoichiometry sufficient for neutralization (see below) (Nelson et al., 2008). The structural basis for this phenomenon is not yet clear and may differ for antibodies that recognize distinct classes of epitopes. Some antibodies may neutralize partially mature virions more efficiently because they recognize epitopes that are exposed on a given oligomeric form of E protein only when prM remains bound. Alternatively, the presence of prM-E heterotrimers on infectious "partially mature" virions may alter the dynamics of the ensemble of conformations displayed by an individual virion. That the polyclonal response of ~50% of human recipients of two different candidate WNV vaccines had substantially less neutralizing activity against mature WNV virions as compared to partially mature virions highlights the importance of understanding the composition of virus populations produced by infected humans and mosquitoes in vivo (Nelson et al., 2008).

Stoichiometric Models of Neutralization

The number of antibodies required to bind a virion and neutralize infectivity has been studied extensively. Generally, two models are favored. "Single-hit" models describe neutralization after

engagement of the virion with a single antibody (Dulbecco et al., 1956). Historically, this model is based on the observation that the neutralization of some viruses appeared to be a first-order reaction, indicating that multiple antibodies are not required to inactivate infectivity (which would be suggested by a lag during kinetic studies). Mechanistically, these models propose that engagement of the virion at "critical sites" is sufficient to cause irreversible conformational changes in the virion that render it noninfectious. In contrast, a "multiple-hit" model of neutralization postulates that virus inactivation occurs as a function of the number of antibodies bound to the virion (Burnet et al., 1937; Della-Porta and Westaway, 1978). In this case, neutralization is reversible and occurs when an individual virion is engaged with a stoichiometry that exceeds a particular threshold. The threshold number of antibodies required for neutralization of different virus classes has been investigated and varies significantly. While the factors that determine the stoichiometric threshold for a given virus are not clear, Burton and colleagues propose that the requirements for neutralization is a reflection of the number of antibodies required to "coat" the surface area of the virus particle (Burton et al., 2001). While the stoichiometric requirements for neutralization remains somewhat controversial and may differ between viral systems or among structurally distinct epitopes, understanding how antibodies block infection remains an important goal.

The existence of high-resolution structural data and the availability of large numbers of well-characterized mAbs identifies flaviviruses as a promising system to investigate models for, and mechanisms of, antibody-mediated neutralization. Several lines of evidence suggest that flavivirus neutralization by antibodies is a multiple-hit phenomenon including (1) the relationships between the affinity of mAbs for the virion and their neutralization activity in vitro, (2) the demonstration that not all antibodies can bind the virion with a stoichiometry sufficient for neutralization, (3) the ability of complement to augment neutralization, and (4) the ability of antibodies to enhance flavivirus infection of Fc-γ-receptor bearing cells (Della-Porta and Westaway, 1978; Gromowski and Barrett, 2007; Gromowski et al., 2008; Mehlhop et al., 2005; Nelson et al., 2008; Pierson et al., 2007). Using the WNV E-DIII-LR-specific mAb E16, we have investigated the number of antibodies bound to the virion when it is neutralized (Pierson et al., 2007). Analysis of the relationship between the strength of binding and neutralization potential of several DIII-LR-specific antibodies suggest that neutralization requires engagement of only a modest fraction of the accessible epitopes on the virion. In agreement, complementary genetic approaches indicate that neutralization occurs when ~25% of the 120 accessible E-DIII-LR epitopes on WNV are engaged by antibody, corresponding to roughly 30 mAbs (Pierson et al., 2007) (Figure 3). Of interest, this estimate agrees with the predictions of the "coating theory" (Burton et al., 2001). Thus, in quantitative terms, the potent inhibitory activity of E16 is explained by its high affinity and the relatively small fraction of E-DIII-LR epitopes on the virion that must be bound by antibody in order to neutralize virus infectivity.

However, not all high-affinity antibodies neutralize flavivirus infection at low concentrations or by binding a small fraction of the epitopes that are accessible on the virion. In fact, many mAbs neutralize infection at concentrations that saturate all the accessible epitopes on the virion (Nelson et al., 2008; Pierson et al., 2007). Because epitopes are not displayed in an identical fashion on E proteins associated with the three distinct symmetry environments of pseudoicosahedral mature flaviviruses (Figure 2E), the total number of accessible binding sites on the mature flavivirus may differ between structurally distinct epitopes. Thus, differences in the fraction of epitopes a particular antibody must bind in order to exceed stoichiometric requirements for neutralization (defined as epitope occupancy) reflect complexities that arise from the arrangement of E proteins on the mature virion. Many antibodies with high-occupancy requirements for neutralization recognize epitopes that are predicted to be poorly accessible on the mature virions. Presumably, when few epitopes are available on the average virion, there is a requirement for

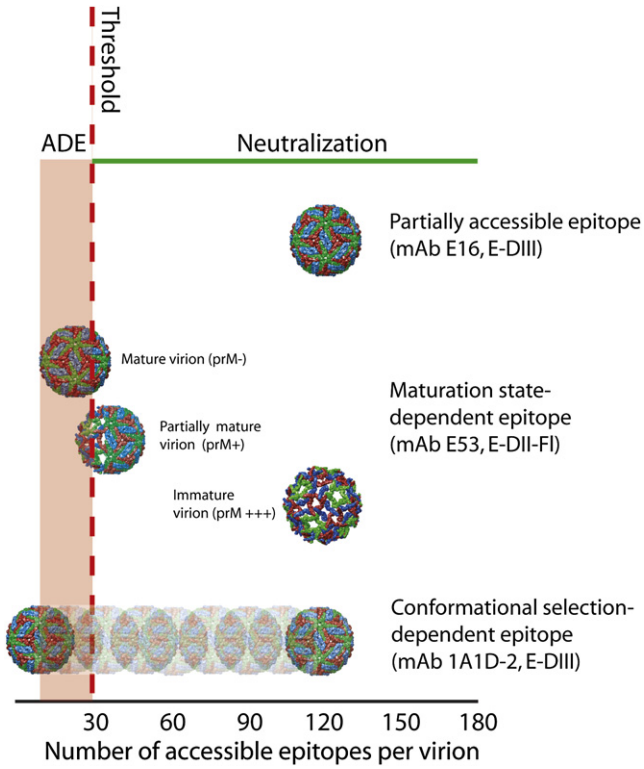

Figure 3. A Model of the Stoichiometric Requirements for Flavivirus Neutralization

Neutralization of flavivirus infection is a multiple-hit phenomenon that requires engagement of the virion by antibody with a stoichiometry that exceeds a threshold (modeled as 30 mAbs based on studies with WNV E-DIII-LR-specific mAbs; red dashed line) (Pierson et al., 2007). Whether an individual antibody can dock on the virion with a stoichiometry sufficient to exceed this threshold depends on its affinity for viral antigens and the total number of accessible epitopes on the average virion (shown schematically on the x axis). Epitopes that are differentially accessible on virions as a function of the extent of virion maturation or the structural dynamics of the virus particle add to the complexity of the model (shown schematically for maturation-dependent epitopes and those that bind selective conformations, respectively). Neutralization via highly accessible determinants can be achieved by engagement to a relatively low occupancy, whereas antibodies that bind cryptic determinants must bind a larger fraction of them. Not all epitopes appear to exist on the average virion at levels that exceed this threshold. Engagement of the virion with a stoichiometry below this threshold may support antibody-dependent enhancement of infection. Virion images were created using Chimera (http://www.cgl.ucsf.edu/chimera) or obtained from the VIPERdb Virus Particle Explorer (http://viperdb.scripps.edu) (Shepherd et al., 2006).

antibodies to bind a larger fraction of them to exceed the stoichiometric threshold required for neutralization. Indeed, not all antibodies have the ability to neutralize all virions in a heterogeneous population (composed of immature, mature, and partially mature virions in unknown proportions) (Oliphant et al., 2006). This "resistant" fraction reflects the existence of individual virions that do not display a sufficient number of epitopes to exceed the threshold even when fully occupied by a given antibody (Nelson et al., 2008; Pierson et al., 2007). Thus, coupled with antibody affinity for the virion, epitope accessibility is a critical parameter that governs the occupancy requirements for neutralization.

Additional studies are required to understand the details that define the stoichiometric requirements of flavivirus neutralization. Our estimates of the stoichiometric threshold of neutralization arise from studies with WNV DIII-LR-specific mAbs. Expanding this analysis to other structural epitopes will be of significant value and provide an interesting test of the "coating theory." However, this is technically quite challenging because, for many of these other epitopes, the total number of accessible sites on the average virion is either variable (in the case of epitopes sensitive to the prM content of virions), or cannot be determined using existing structural approaches (e.g., epitopes exposed only on conformations of the virion that are underrepresented in the dynamic ensemble of possible conformations of the virion) (Figure 3). It is also unclear if the stoichiometric requirements for neutralization differ between antibodies that neutralize infection via different mechanisms (see below). Finally, whether the stoichiometric requirements for neutralization by mAbs that capture potentially transient conformations of the virion (e.g., 1A1D-2) differ from our estimates obtained with E16 is of significant interest and may provide an opportunity to explore whether one model for neutralization ("single-hit" versus "multiple-hit") is appropriate for all antibodies that recognize a particular virus.

Implications for Antibody-Mediated Enhancement of Infection

Insight into the stoichiometric requirements for neutralization provides a biochemical rationale to understand antibody-mediated enhancement of infection (ADE). ADE describes an opsonic phenomenon by which antibody or antibody plus complement increases the efficiency or duration of the interaction between virus and target cells by virtue of interactions with Fc-γ- or complement receptors (Cardosa et al., 1983; Gollins and Porterfield, 1984). Our analyses of more than 100 mAbs raised against WNV and DENV suggest that all antibodies that bind the virion and neutralize infection have the potential to promote ADE in vitro, irrespective of their epitope specificity (Oliphant et al., 2006; Pierson et al., 2007). Detailed functional studies revealed that the concentrations of antibody that promote maximal enhancement of infection in vitro were very similar to those that neutralize half the virions in a given experiment (Pierson et al., 2007). This reflects the fact that neutralization and ADE are two phenomena related by the number of antibodies bound to the virion. When half of the virions are engaged by antibody with a stoichiometry sufficient to inactivate virus infection, the other half are not and remain infectious. Thus, at the upper limit, the number of antibodies that can promote ADE is defined by the stoichiometric threshold of neutralization. The minimum number of antibodies required for ADE, based on experimental data, appears to be approximately half the number required for neutralization (Pierson et al., 2007).

Despite intense interest, the characteristics of antibodies that are likely to enhance infection in vivo have not been determined. The threshold requirement for neutralization central to the "multiple-hit" model of neutralization suggests that individual virions can be bound by antibody with a stoichiometry that is not sufficient for neutralization. This provides an important framework upon which to consider the enhancing potential of antibodies that recognize different classes of epitopes. The ability of potent neutralizing antibodies (like the E-DIII-LR-specific mAb E16) to completely block infection at high concentrations of antibody limits their potential for ADE. However, antibodies that recognize poorly accessible epitopes (such as the E-DII-FL) are often incapable of neutralizing all members of a heterogeneous population of virions, even under saturating conditions of antibody. Under these conditions, the fraction of virions resistant to neutralization may be bound by antibody with a stoichiometry that promotes ADE. Thus, accessibility of the epitope is a critical factor for assessing the potential for a class of antibody to promote ADE (Nelson et al., 2008). Beyond stoichiometric considerations, other factors may modulate the potential for antibodies to enhance infection including the glycosylation state of the mAb, its isotype, and the presence of serum factors such as complement (Mehlhop et al., 2007). Furthermore, ADE by anti-flavivirus antibodies that promote more efficient infection through non-Fc receptor-dependent pathways, possibly by enhancing attachment or entry through distinct mechanisms, may be uniquely regulated (Huang et al., 2006).

Mechanisms of Neutralization

Many steps in the flavivirus virus entry pathway may be blocked by antibody including (1) virion attachment to the cell, (2) interaction of virions with host factors required for internalization or fusion, and (3) E protein-mediated conformational changes that drive membrane fusion. At present, an incomplete picture of how flaviviruses interact with permissive cells limits our understanding of mechanisms of neutralization. Many of the neutralizing antibodies characterized using other viral systems inhibit infection by interfering with required interactions between the viral glycoproteins and cellular receptors. While several molecules that promote more efficient flavivirus attachment (e.g., DC-SIGN, DC-SIGNR, heparin sulfate) have been characterized, cellular receptors required for flavivirus entry have not been identified (Anderson, 2003). While the relevant interactions are unclear, inhibition of flavivirus attachment by some mAbs and flavivirus-immune sera contributes to virus neutralization (Crill and Roehrig, 2001; He et al., 1995; Nybakken et al., 2005). Additionally, antibodies that inhibit viral fusion have also been characterized (Gollins and Porterfield, 1986; Roehrig et al., 1998; Stiasny et al., 2007). Stiasny and colleagues reported that some but not all antibodies had the capacity to block fusion of TBEV with synthetic liposome (Stiasny et al., 2007). A similar result has been obtained using the WNV-specific mAb E16 (B.S. Thompson, B.J.S. Moesker, J.M. Smit, J. Wilschut, M.S.D., and D.H.F., unpublished data). In the latter case, molecular docking studies of E16 Fab fragments on mature virions

identify steric conflicts that should prevent the conformational and organization changes in E proteins that drive fusion at low pH (Kaufmann et al., 2006; Nybakken et al., 2005).

The mechanisms by which a given antibody neutralizes infection may depend upon circumstance, as single antibodies may block more than one step in the viral entry pathway. For example, the mAb E16 is capable of blocking both viral fusion and attachment of WNV to cells (Nybakken et al., 2005). Whether an ability to neutralize infection via multiple mechanisms is a common feature of the most potent antibodies remains unclear. Thus, while several steps in the flavivirus entry pathway that may be inhibited by antibody have been cataloged, future studies are required to identify factors that regulate these distinct neutralization mechanisms.

The Role of Fc-Dependent Effector Functions of Antibodies in Protection against Flavivirus Infection

Beyond direct virus neutralization, antibody binding to virions or virus-infected cells can trigger protective Fc-dependent antiviral activities through complement activation or Fc-γ-receptor-mediated immune complex clearance mechanisms. Fc-γ receptors are expressed on a wide variety of immune cells and belong to a multigene immunoglobulin-like superfamily that can activate or inhibit immune responses depending on their cytoplasmic domain or association with specific signaling molecules (Nimmerjahn and Ravetch, 2008). Recent studies indicate that interaction of the Fc region of antibodies with activating Fc-γ receptors can augment antibody protection against flavivirus infection in vivo. Mice lacking all activating Fc-γ receptors required significantly higher doses of a neutralizing E protein-specific mAb to maintain equivalent levels of protection against lethal WNV infection (Oliphant et al., 2006), and Fab_2 fragments of a neutralizing mAb were less protective in mice (Gould et al., 2005).

Ab-Mediated Complement Activation and Protection

The complement system is a family of ~30 serum and cell-surface proteins that recognize and clear pathogens and activate inflammatory responses (Carroll, 2004). The complement cascade protects generally against viral infection through lysis of virions or infected cells, release of proinflammatory peptides, opsonization and neutralization of viruses, targeting to complement receptor-expressing cells, and facilitating antigen presentation and immune cell priming. Opsonization of enveloped RNA viruses with classical pathway complement components (C1q, C4b, and C3b) in an antibody-dependent manner can inhibit receptor attachment or viral fusion and promote the formation of C5b-C9 membrane attack components that induce virolysis. Indeed, the absence of classical pathway complement components leads to more severe primary WNV infection (Mehlhop et al., 2005). More recent studies demonstrate that for the specific IgG subclasses that bind C1q avidly (mouse IgG2a and 2b; human IgG1 and 3), complement augments antibody-mediated neutralization of WNV and DENV in vitro and increases the functional potency of a strongly neutralizing anti-WNV antibody in mice in an IgG-subclass-specific manner (E. Mehlhop, S. Nelson, C.A. Jost, S. Johnson, D.H.F., M.S.D., and T.C.P., unpublished data). In agreement, a mouse IgG2a isotype-switch variant of an E protein-specific mAb against YFV protected mice more efficiently from lethal infection than the parental IgG1 (Schlesinger and Chapman, 1995).

Implications for the Development of Novel Flavivirus Vaccines

The characteristics of a protective humoral immune response elicited by natural infection or vaccination have not been defined in molecular terms. Current in vitro markers and assays are not reliable surrogates of protection in vivo. The root of this problem may be that existing approaches cannot measure the contribution of relevant specificities or functional properties of a humoral response against the backdrop of a complex and extensively crossreactive polyclonal antibody response. For example, neutralizing titers induced by the tetravalent vaccines against DENV as determined using the standard assay (plaque reduction neutralization test) have not completely correlated with protection in challenged non-human primates (Blaney et al., 2005). Efforts to elucidate the structural, functional, and biophysical correlates of antibody-mediated protection will facilitate the identification of protective epitopes in vivo, inform the development of assays capable of specifically measuring responses to these determinants, and guide the refinement of vaccination strategies to focus and augment the humoral response.

Epitope-Specific Diagnostics

The development of DENV vaccines is actively progressing; the first generation of tetravalent candidate DENV vaccines will soon be employed in large clinical trials in DENV endemic regions to evaluate safety and efficacy in humans. However, the tools to quantitatively describe the features of protective and nonprotective humoral responses are not yet available. For example, it remains unclear if four distinct type-specific responses are required to achieve protection against all four serotypes of DENV. Furthermore, how a protective repertoire of antibody changes over time and with repeated exposure to DENV antigens may provide new insights into optimizing vaccine delivery schedules and inform our understanding of the durability of a protective response. To this end, the development of epitope-specific measures of anti-flavivirus antibodies will clarify the levels and functional contribution of different classes of antibody in vivo. For example, the epitope specificity and functional activity of the natural antibody response against WNV in mice, horses, and humans has been investigated using loss-of-function E proteins, E-DIII, or viral variants that lacked the capacity to bind antibodies specific for the E-DIII-LR epitope (Oliphant et al., 2007; Sanchez et al., 2007). While E16-like antibodies can be detected in convalescent serum, the functional role for E-DIII-LR-specific antibodies during the response to WNV infection appears modest despite their potent neutralizing activity. Similar results were observed during the analysis of the humoral response of recipients of two different candidate WNV vaccines (Nelson et al., 2008). In contrast, a significant fraction of E protein-specific B cells appear to produce antibodies specific for the E-DII-FL (Throsby et al., 2006), and antibodies against this determinant are readily detected in serum. Using a similar approach, mutations in the fusion loop have improved the accuracy of serum diagnostic assays for DENV, JEV, and SLEV by eliminating detection of the highly crossreactive fusion loop antibodies (Chiou et al., 2008, and references within). As a more detailed understanding of the structural basis of antibody recognition and neutralization of flaviviruses emerges, it may be possible to generate high-throughput epitope-based diagnostic assays with a series of E protein variants that defines serotype specificity and

functional classes of neutralizing antibodies after infection or vaccination.

Novel Flavivirus Immunogens

Induction of neutralizing antibodies is critical for the efficacy of many antiviral vaccines, and the structural and functional characteristics of the most potent anti-flavivirus neutralizing antibodies are becoming clear. Beyond simply eliciting antibodies that recognize viral antigens with high affinity, the goal of recombinant immunogens should be to target accessible determinants on the mature virion that allow for neutralization at low occupancy and reduce the potential for ADE. Existing vaccination strategies may produce strong anti-flavivirus antibody responses against viral structural proteins, as measured by ELISA, that do not correspond to high neutralizing titers in vitro or in vivo protection (Despres et al., 2005; Throsby et al., 2006). This disparity may be due to the repertoire of antibodies elicited by a particular immunogen. Our improved understanding of the structural and mechanistic correlates of antibody protection may inform novel strategies for modifying E proteins to preserve specific neutralizing epitopes and subtract or mask immunodominant poorly neutralizing epitopes that may have the potential to support ADE in vivo. The feasibility of this approach has been established by studies that direct the immune response away from non-neutralizing antibodies against HIV gp120 (Pantophlet and Burton, 2003). However, the design of a tailored flavivirus immunogen will likely require an iterative process. One primary decision will be whether to use the whole ectodomain of the envelope protein or E-DIII alone as the immunogen. While E-DIII contains the strongly neutralizing lateral ridge epitope, it also displays a highly crossreactive immunodominant non-neutralizing epitope in the AB loop (Sukupolvi-Petty et al., 2007). Furthermore, strongly neutralizing and highly protective antibodies in DI have recently been described against DENV and JEV (Goncalvez et al., 2008; Lai et al., 2007); it is not clear whether responses to these epitopes are functionally important in vivo. Additionally, domains I and II may contain important class II MHC-dependent epitopes that are necessary for T cell-directed affinity maturation. Indeed, recent studies indicate that neutralizing antibody responses against a given epitope of vaccinia virus is augmented by coimmunization with a linked immunodominant class II MHC-restricted viral peptide (Sette et al., 2008). While the development of novel immunogens capable of eliciting (or boosting) type-specific or protective responses are at early stages, these efforts are informed by continued advances in our basic understanding of the mechanisms that govern antibody potency in vitro and in vivo.

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